Successful capacitation and homologous fertilization *in vitro* in *Calomys musculinus* and *Calomys laucha* (Rodentia – Sigmodontinae)

A. Lasserre*, E. Cebral and A. D. Vitullo

Centro de Estudios Farmacológicos y Botánicos (CEFyBO-CONICET), Serrano 669. (1414), Capital Federal Argentina

Small South American rodents of the genus *Calomys* have been used extensively for virology and ecological research. Previous studies have demonstrated that *Calomys musculinus* and *Calomys laucha* have a relatively short oestrous cycle and that superovulation and parthenogenetic activation can be induced. The purpose of this study was to determine the requirements for *in vitro* manipulation of the male gamete and *in vitro* fertilization. Two culture media and different concentrations of spermatozoa were tested for their ability to support sperm motility, hyperactivation and the acrosome reaction. The ability of capacitated *Calomys* spermatozoa to penetrate zona-free hamster eggs was also evaluated. *In vitro* fertilization was assessed by examining attachment and binding to the zona pellucida, second polar body extrusion, pronucleus formation and the fertilizing sperm tail. The results of the study showed that: (i) Tyrode's albumin lactate pyruvate (TALP) medium was more effective than T6 medium for maintaining sperm motility *in vitro*; (ii) hyperactivation was achieved with TALP but not with T6; (iii) the acrosome reaction was easily distinguished by light microscopy and depends on time and sperm concentration; (iv) capacitated spermatozoa are able to penetrate zona-free hamster eggs; and (v) superovulated oocytes can be fertilized *in vitro*. This is the first report of capacitation and *in vitro* fertilization for *Calomys* sp. These results provide opportunities to use *C. musculinus* and *C. laucha* as new laboratory animals for research into reproductive biology.

Introduction

Calomys, a South American mouse genus, occurs in a variety of habitats including mountain grassland, brushy areas and forest fringes in Argentina, Bolivia, Brazil, Paraguay, Peru, Uruguay and Venezuela (Reig, 1984). Their morphological and ecological diversity make it interesting to consider the presumptive variations in their behaviour. *Calomys* has epidemiological importance as a natural reservoir of arena viruses, hanta viruses and protozoans hazardous to humans (Weissenbacher *et al.*, 1987, 1990; Mills *et al.*, 1994; Childs *et al.*, 1995). Its several unique characteristics have resulted in adoption of this genus for studies in various biomedical fields. *Calomys musculinus* and *Calomys laucha* are easy to breed, have large litter sizes and ovulate spontaneously through the year. Attempts have been made to introduce superovulation and parthenogenetic activation and to culture preimplantation embryos (Lasserre *et al.*, 1998, 1999a,b). Preliminary results demonstrated that the oocytes

from *C. musculinus* and *C. laucha* have different requirements compared with those of conventional rodents.

Studies to characterize the biological aspects of reproduction require a successful IVF procedure in the species of choice. In this connection, it is essential to evaluate sperm motility, capacitation, the acrosome reaction and the subsequent ability for attachment and binding to, and penetration of, the zona pellucida, oocyte activation and pronuclei formation. It is well known that pre-incubated spermatozoa can undergo capacitation in certain artificial media, although the time required for capacitation varies among species (Toyoda *et al.*, 1971; Yanagimachi, 1970, 1972; Niwa and Chang, 1974).

The present study is part of a broader programme on the reproductive biology of these species in an attempt to consolidate their potential usefulness as an experimental model. The study involved examination of different media, sperm concentration, capacitation time, the acrosome reaction and the time required to complete fertilization. A heterologous sperm penetration assay (Yanagimachi, 1984) was also performed, which evaluates sperm capacitation and the acrosome reaction. On the basis of the results, a successful *in vitro* fertilization procedure was developed in *C. musculinus* and *C. laucha*.

*Correspondence address: Instituto de Biología y Medicina Experimental (IBYME-CONICET) Vuelta de Obligado 2490, (1428) Capital Federal, Argentina.

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Materials and Methods

Animals

Calomys laucha has been bred in this laboratory since 1993; the colony originates from mice of the Universidad de Córdoba. *Calomys musculinus* has been bred in this laboratory since 1992; the colony was initiated from two males and one female captured at Donovan, province of San Luis, Argentina. General husbandry and breeding conditions were as described by Hodara *et al.* (1984, 1989). Female golden hamsters (6–8 weeks old) were provided by Dr C. Barros, Pontificia Universidad Católica de Chile. The animals were maintained under controlled conditions of 14 h light:10 h dark and were fed with a commercial mouse chow. Food and water were provided *ad libitum*. Females and males used for experimentation were identified at weaning and maintained in groups of two to three animals until used.

Culture media

T6 was prepared according to the method of Wood (1987) supplemented with 5% BSA (Sigma, St Louis, MO). Tyrode's albumin lactate pyruvate (TALP) medium was prepared according to the method of Bavister and Yanagimachi (1977) supplemented with 5% BSA. M2 medium (Fulton and Whittingham, 1978) contained 4% BSA. All media were sterilized by filtering. Media were equilibrated under 5% CO₂ in air for at least 2 h at 37°C.

Collection of spermatozoa

Sexually mature (3 months old) males were killed by cervical dislocation. The epididymides were excised and washed in medium to remove any traces of blood. Cauda epididymides were placed in a 0.5 ml drop of medium and covered with mineral oil. The tubules were cut with a pair of sharp iridectomy scissors to allow the dense mass of spermatozoa to flow out freely into medium for 5 min in an atmosphere of 5% CO₂ in air at 37°C. The concentration of spermatozoa was determined using a haemocytometer.

Sperm capacitation

Motility, hyperactivation and the acrosome reaction. Sperm suspensions were incubated for 2 h (5% CO₂ in air at 37°C) in either T6 or TALP at concentrations of $1-2 \times 10^7$ and $1-2 \times 10^8$ spermatozoa ml⁻¹. Motility, hyperactivation and the acrosome reaction were evaluated throughout the incubation period (0, 30, 60, 90 and 120 min). The pattern of hyperactivation was analysed by observation under an inverted microscope and was evaluated for the total motile population. The proportion of acrosome-reacted and acrosome-intact spermatozoa was evaluated directly by light microscopy.

Sperm penetration assay. A heterologous sperm penetration

assay was performed using zona-free hamster eggs according to the method of Chaudhuri and Yanagimachi (1984) to evaluate sperm fertilizing ability, including capacitation and the acrosome reaction. This part of the study was conducted at the Pontificia Universidad Católica de Chile. Briefly, 2–3 month-old female hamsters were superovulated by injection of 50 iu equine chorionic gonadotrophin (eCG; Folligon, Intervet) on the morning of day 1 of the oestrous cycle followed by injection of 50 iu human chorionic gonadotrophin (hCG; Choloron, Intervet) 54–58 h later. Between 15 and 16 h after hCG injection, oviducts were excised and placed in a dish with M2 medium + 4% BSA. The oocyte–cumulus complexes in the oviduct were released from the ampulla using a needle. After treatment with 0.1% (w/v) hyaluronidase, cumulus-free hamster eggs were isolated and treated in M2 medium (pH 7.4) containing 0.05% (w/v) trypsin for 2 min to remove the zona pellucida. Sperm were incubated to allow capacitation in TALP medium at a concentration of $1-2 \times 10^7$ or $1-2 \times 10^8$ spermatozoa ml⁻¹. After 90 min of pre-incubation, the eggs were inseminated with either $1-2 \times 10^7$ or $1-2 \times 10^6$ spermatozoa ml⁻¹ and incubated at 37°C in 5% CO₂ in air. After 3–4 h of incubation, the eggs were fixed with 2.5% (v/v) formalin and stained with 10 µg Hoescht 33342 ml⁻¹ (Sigma) in Hepes-buffered medium (pH 7.4) and examined under a phase-contrast fluorescence microscope. Sperm penetration was evaluated by observation of swelling of the sperm head and decondensation. The number of eggs penetrated and the mean number of spermatozoa per penetrated egg were recorded. Five replicates were carried out for each experiment.

In vitro fertilization

Source and collection of eggs. Mature *C. musculinus* and *C. laucha* females (2 months old) were induced to superovulate as described by Lasserre *et al.* (1998, 1999a). Briefly, eCG and hCG were injected i.p. 48 h apart, 12 and 15 iu, respectively, for *C. musculinus* and 8 and 8 iu, respectively, for *C. laucha*. Females were killed by cervical dislocation 14–15 h after hCG injection. Cumulus-enclosed eggs were released by puncturing the ampulla into M2 medium containing 4% BSA and transferred into droplets (100 µl) of fresh medium under mineral oil in a plastic Petri dish (35 mm diameter).

In vitro insemination. After pre-incubation, the spermatozoa were transferred into 100 µl TALP medium containing a cumulus–oocyte complex and incubated for 6 h at 37°C in 5% CO₂ in air. The following combinations were tested: (i) sperm capacitation with $1-2 \times 10^7$ spermatozoa ml⁻¹ and insemination with $1-2 \times 10^5$ or $1-2 \times 10^6$; (ii) sperm capacitation with $1-2 \times 10^8$ spermatozoa ml⁻¹ and insemination with $1-2 \times 10^6$ or $1-2 \times 10^7$ spermatozoa ml⁻¹.

Examination of IVF events. The eggs were examined at 2.5, 5.0 and 6.0 h after insemination. Whole mount egg preparations were examined under a phase-contrast microscope after they were fixed with 2.5% (w/v) glutaraldehyde for 20 min at room temperature and stained

with Hoescht 33258 ($10 \mu\text{g ml}^{-1}$) At 2.5 and 5.0 h after insemination, sperm attachment was examined under the microscope and the number of spermatozoa attached to each egg was determined. After eggs were washed twice and pipetted in and out of a fine-bore pipette about ten times, the number of spermatozoa that remained bound to each egg was recorded, and the eggs were examined for evidence of sperm penetration.

In a second series of experiments, eggs were examined about 6 h after insemination for extrusion of the second polar body (activated oocytes), for female and male pronuclei and for the presence of a fertilizing sperm tail.

Statistical analysis

Statistical analyses were performed by means of the Instat program (Graphpad Software, San Diego, CA). ANOVA or the chi-squared test was used for comparisons between groups. Differences resulting in a P value < 0.05 were considered significant.

Results

Effect of medium and sperm concentration on sperm motility

The effect of incubation of *Calomys* spermatozoa in T6 or TALP medium for 2 h is shown (Fig. 1). In *C. musculinus* (Fig. 1a) between 30 and 120 min of incubation, the percentage of motility decreased in T6 medium more significantly than in TALP medium at both sperm concentrations ($1-2 \times 10^7$ and $1-2 \times 10^8$ spermatozoa ml^{-1}). In *C. laucha* (Fig. 1b), the same pattern of motility was found for both media ($P < 0.001$). Exposure to T6 induced sperm agglutination (head to head) in both species (data not shown).

Effect of medium and sperm concentration on hyperactivation

Cauda epididymal spermatozoa were very active immediately after they were suspended in T6 or TALP, showing a very fast progressive linear motility. The motility pattern of *Calomys* spermatozoa changed during the course of incubation and it was possible to recognize two other patterns within the hyperactivated group: 'circular', in which spermatozoa moved in circles, and 'wriggling', in which spermatozoa showed rapid wriggling and swam in concentric circles.

In *C. musculinus*, the spermatozoa incubated with T6 medium showed only linear progressive motility and never developed the hyperactivation pattern. In TALP medium, the percentage of spermatozoa showing hyperactivation increased with incubation time up to 90 min and this value was maintained up to 120 min (Fig. 2a), despite the different sperm concentrations ($P < 0.001$). In *C. laucha* (Fig. 2b), the percentage of hyperactivation increased progressively up to 60 min in TALP medium at both sperm concentrations, but at

Fig. 1. Effect of medium and sperm concentration on sperm motility in (a) *Calomys musculinus* and (b) *Calomys laucha*. *Calomys* spermatozoa were capacitated for 2 h in Tyrode's albumin lactate pyruvate (TALP) medium at $1-2 \times 10^8$ ($\bullet$) or $1-2 \times 10^7$ ($\blacktriangle$) spermatozoa ml^{-1} , or in T6 medium at $1-2 \times 10^8$ ($\blacksquare$) or $1-2 \times 10^7$ ($\blacklozenge$) spermatozoa ml^{-1} . Percentage motility (and SEM) were evaluated for ten males and were analysed by Student's t test. (a) *C. musculinus*: TALP + 10^7 and 10^8 spermatozoa ml^{-1} at 120 versus 30 min of incubation: $P < 0.01$. T6 + 10^7 and 10^8 spermatozoa ml^{-1} at 120 versus 30 min: $P < 0.001$. At 30 min of incubation: TALP + 10^7 versus T6 + 10^7 spermatozoa ml^{-1} and TALP + 10^8 versus T6 + 10^8 spermatozoa ml^{-1} : $P < 0.001$. At 120 min of incubation: TALP + 10^7 versus T6 + 10^7 spermatozoa ml^{-1} and TALP + 10^8 versus T6 + 10^8 spermatozoa ml^{-1} : $P < 0.001$. For TALP + 10^7 and 10^8 spermatozoa ml^{-1} at 120 versus 30 min: $P < 0.01$. For T6 + 10^7 and 10^8 spermatozoa ml^{-1} at 120 versus 30 min: $P < 0.001$. (b) *C. laucha*: the same groups were analysed: $P < 0.001$ (Student's t test).

$1-2 \times 10^7$ spermatozoa ml^{-1} the hyperactivation rate was lower at 90 and 120 min than at $1-2 \times 10^8$ spermatozoa ml^{-1} . In T6 medium, a high percentage of spermatozoa became hyperactivated by 30 min and this value remained constant up to 120 min, at which time the percentage was significantly lower than with TALP medium ($P < 0.001$). The development of a particular motility pattern was time-dependent. Spermatozoa showing linear motility were the most predominant at 0 min, but gradually decreased in number, and the proportion of spermatozoa showing a circular motion increased by 2 h. During incubation, there was a reduction in the diameter of the circular trajectory, and the proportion of spermatozoa showing the wriggling movement was small ($< 4\%$).

Fig. 2. Effect of medium and concentration on sperm hyperactivation in (a) *Calomys musculus* and (b) *Calomys laucha*. *Calomys* spermatozoa were capacitated for 2 h in Tyrode's albumin lactate pyruvate (TALP) medium at $1-2 \times 10^8$ (●) or $1-2 \times 10^7$ (▲) spermatozoa ml^{-1} , or in T6 medium at $1-2 \times 10^8$ (■) or $1-2 \times 10^7$ (◆) spermatozoa ml^{-1} . Percentages of hyperactivation (and SEM) were evaluated for ten males and analysed by Student's *t* test. (a) *C. musculus*: 30 and 120 min of incubation and the same sperm suspensions as in Fig. 1: $P < 0.001$. (b) *C. laucha*: at 30 min of incubation: TALP + 10^7 versus T6 + 10^7 spermatozoa ml^{-1} : $P < 0.001$; TALP + 10^8 versus T6 + 10^8 spermatozoa ml^{-1} : $P < 0.01$. At 120 min of incubation: TALP + 10^7 versus T6 + 10^7 spermatozoa ml^{-1} and TALP + 10^8 versus T6 + 10^8 spermatozoa ml^{-1} : $P < 0.001$.

Effect of medium and sperm concentration on the acrosome reaction

C. musculus spermatozoa have a hooked head, a roughly polygonal nucleus and a flat head base, and the tail inserts eccentrically and ipsilateral to the hook. In contrast, *C. laucha* spermatozoa do not have a hooked head, the nucleus is grossly pyriform but asymmetrical, and the tail inserts centrally in the flat base of the head. It is very easy to distinguish acrosome-intact from acrosome-reacted spermatozoa by light microscopy because the acrosome reaction involves a visible change in head morphology.

The pattern of spontaneous acrosome reactions throughout the incubation period is shown (Fig. 3). In *C. musculus* (Fig. 3a) at a concentration of 10^8 spermatozoa ml^{-1} , a maximum percentage of acrosome reactions developed by 60 min in TALP medium ($P < 0.001$). Under the other conditions (10^7 spermatozoa ml^{-1} in TALP and 10^7 and

Fig. 3. Effect of the medium and sperm concentration on the acrosome reaction in (a) *Calomys musculus* and (b) *Calomys laucha*. *Calomys* spermatozoa were capacitated for 2 h in Tyrode's albumin lactate pyruvate (TALP) medium at $1-2 \times 10^8$ (●) or $1-2 \times 10^7$ (▲) spermatozoa ml^{-1} , or in T6 medium at $1-2 \times 10^8$ (■) or $1-2 \times 10^7$ (◆) spermatozoa ml^{-1} . Percentages of acrosome reaction (and SEM) were evaluated for ten males and analysed by Student's *t* test. (a) *C. musculus*: for 60 and 120 min of incubation: TALP + 10^7 versus T6 + 10^7 spermatozoa ml^{-1} and TALP + 10^8 versus T6 + 10^8 spermatozoa ml^{-1} : $P < 0.001$. (b) *C. laucha*: for 30, 60 and 90 min of incubation the three groups are significantly different: $P < 0.001$. For 120 min of incubation: TALP + 10^8 versus T6 + 10^8 spermatozoa ml^{-1} : $P < 0.001$; TALP + 10^7 versus T6 + 10^7 spermatozoa ml^{-1} : not significant; T6 + 10^8 versus T6 + 10^7 spermatozoa ml^{-1} : $P < 0.05$.

10^8 spermatozoa ml^{-1} in T6), the acrosome reaction increased significantly throughout the 120 min of incubation ($P < 0.001$), and there was no significant difference between 60 and 120 min when 10^8 spermatozoa ml^{-1} were incubated in TALP medium. In *C. laucha* (Fig. 3b), the greatest acrosome reaction rate occurred at 90 min of incubation in both groups in TALP and in T6 media, but although the percentage of acrosome reactions increased from 90 to 120 min with 10^7 spermatozoa ml^{-1} in TALP or T6 medium, the percentages were significantly lower with 10^8 spermatozoa ml^{-1} incubated in TALP or T6 medium at 120 min.

Sperm penetration assay

The results presented above indicate that *Calomys* spermatozoa require TALP medium for optimal motility and hyperactivation and also for the acrosome reaction. The effect of the sperm concentration on the ability of *Calomys* spermatozoa to penetrate zona-free hamster eggs was also

Table 1. Ability of *Calomys musculus* and *Calomys laucha* spermatozoa incubated in Tyrode's albumin lactate pyruvate medium to penetrate zona-free hamster eggs

Species	Sperm concentration at pre-incubation/ <i>in vitro</i> insemination (spermatozoa ml ⁻¹)	Number of eggs penetrated (%)	Number of penetrating spermatozoa (mean $\pm$ SD)
<i>C. musculus</i>	1–2 $\times$ 10 ⁸ /1–2 $\times$ 10 ⁷	26/115 (22.6)***	6.08 $\pm$ 3.5 ^a
	1–2 $\times$ 10 ⁷ /1–2 $\times$ 10 ⁶	102/120 (85.0)	12.08 $\pm$ 4.15
<i>C. laucha</i>	1–2 $\times$ 10 ⁸ /1–2 $\times$ 10 ⁷	21/114 (18.4)***	11.1 $\pm$ 2.8 ^a
	1–2 $\times$ 10 ⁷ /1–2 $\times$ 10 ⁶	118/125 (94.4)	14.11 $\pm$ 3.33

C. musculus: number of eggs penetrated 22.6% versus 85%, *** P < 0.001 (chi-squared test); number of penetrating spermatozoa 6.08 $\pm$ 3.5 versus 12.08 $\pm$ 4.15, ^a P < 0.001 (Student's *t* test).

C. laucha: number of eggs penetrated 18.4% versus 94.4%, *** P < 0.001 (chi-squared test); number of penetrating spermatozoa 11.1 $\pm$ 2.8 versus 14.11 $\pm$ 3.33, ^a P < 0.001 (Student's *t* test).

Fig. 4. Sperm and acrosome morphology of *Calomys* spermatozoa. *C. musculus* spermatozoa (a) acrosome-intact and (b) acrosome-reacted. *C. laucha* spermatozoa (c) acrosome-intact and (d) acrosome-reacted. Scale bar represents 5 μ m.

examined using this medium. In both *Calomys* sperm penetration assays, the highest percentage of penetrated oocytes and greatest number of penetrating spermatozoa were observed when spermatozoa were incubated at a concentration of 10⁷ spermatozoa ml⁻¹ and inseminated at 10⁶ spermatozoa ml⁻¹ compared with incubation at 10⁸ and insemination at 10⁷ spermatozoa ml⁻¹ (P < 0.001) (Table 1).

In vitro fertilization

Attachment and binding to, and penetration of, the zona pellucida by spermatozoa were analysed for both *Calomys* species (Table 2). At 2.5 h of incubation, *C. musculus* eggs had about two to six spermatozoa attached to the zona pellucida after pre-incubation with 10⁷ spermatozoa ml⁻¹ and

Table 2. Zona attachment, binding and penetration of *Calomys musculinus* and *Calomys laucha* spermatozoa using homologous eggs

Species	Sperm concentration		2.5 h		5.0 h	
	Pre-incubation (spermatozoa ml ⁻¹)	<i>In vitro</i> insemination (spermatozoa ml ⁻¹)	Zona attachment	Zona binding	Zona attachment	Zona binding
<i>C. musculinus</i>	1–2 × 10 ⁸	1–2 × 10 ⁷	–	–	+	+
		1–2 × 10 ⁶	–	–	++	+
	1–2 × 10 ⁷	1–2 × 10 ⁶	++	++	+++	++++
<i>C. laucha</i>		1–2 × 10 ⁵	++	++	++	++/+++
	1–2 × 10 ⁸	1–2 × 10 ⁷	–	–	+	+
		1–2 × 10 ⁶	–	–	++	+
	1–2 × 10 ⁷	1–2 × 10 ⁶	++	++	+++	+++
		1–2 × 10 ⁵	++	++	++	+/+++

Results are averages of five different experiments.

Number of spermatozoa per egg: –, no spermatozoa; +, one spermatozoon; ++, two to six spermatozoa; +++, 7–10 spermatozoa; +++++, 11–18 spermatozoa.

Table 3. Egg activation and pronucleus formation during *in vitro* fertilization in *Calomys musculinus* and *Calomys laucha*

Species	Sperm concentration		Fertilized eggs			Unfertilized eggs	
	Pre-incubation (spermatozoa ml ⁻¹)	<i>In vitro</i> insemination (spermatozoa ml ⁻¹)	Number of eggs	Monospermic eggs (%)	Polyspermic eggs (%)	Activated (%)	Fragmented (%)
<i>C. musculinus</i>	1–2 × 10 ⁸	1–2 × 10 ⁷	80	5 (6.25)	1 (1.25)	0 (0)	0 (0)
		1–2 × 10 ⁶	82	15 (18.29)**	2 (2.43)	1 (1.22)	2 (2.43)
	1–2 × 10 ⁷	1–2 × 10 ⁶	78	50 (64.1)*a	1 (1.28)	6 (7.70)	3 (3.85)
<i>C. laucha</i>		1–2 × 10 ⁵	90	44 (48.88)	0 (0)	4 (4.44)	1 (1.11)
	1–2 × 10 ⁸	1–2 × 10 ⁷	84	7 (8.33)	3 (3.57)	1 (1.19)	1 (1.19)
		1–2 × 10 ⁶	81	19 (23.45)***b	2 (2.47)	0 (0)	3 (3.70)
	1–2 × 10 ⁷	1–2 × 10 ⁶	83	58 (69.8)**a	4 (4.83)	3 (3.61)	2 (2.41)
		1–2 × 10 ⁵	82	37 (45.12) ^b	2 (2.44)	2 (2.44)	3 (3.65)

C. musculinus: 1–2 × 10⁷ versus 1–2 × 10⁶ spermatozoa inseminated per ml (*in vitro* insemination): ***P* < 0.01; 10⁶ versus 10⁵ spermatozoa inseminated per ml:

**P* < 0.05. Comparisons between the four cases of *in vitro* insemination: ^a*P* < 0.001 (chi-squared test).

C. laucha: 10⁷ versus 10⁶ spermatozoa inseminated per ml: ****P* < 0.001; 10⁶ versus 10⁵ spermatozoa inseminated per ml: ***P* < 0.01. Comparisons between the four cases of *in vitro* insemination: ^a*P* < 0.001, ^b*P* < 0.01 (chi-squared test).

insemination with 10⁶ or 10⁵ spermatozoa ml⁻¹. At 5 h of incubation, 11–18 spermatozoa were attached and six to ten spermatozoa were bound per egg after pre-incubation with 10⁷ spermatozoa ml⁻¹ and insemination with 10⁶ spermatozoa ml⁻¹. At 5 h of incubation spermatozoa had already started to penetrate the eggs. In *C. laucha*, similar results were observed for the attachment, binding and penetration assays.

At 6 h after insemination, oocyte activation and pronucleus formation were evaluated (Table 3). For both *Calomys* species, the highest percentages of monospermic fertilized eggs were obtained after pre-incubation with 10⁷ spermatozoa ml⁻¹ and insemination with 10⁶ spermatozoa ml⁻¹ (*P* < 0.001).

Discussion

The present study reports a successful IVF procedure for *C.*

musculinus and *C. laucha* using spermatozoa capacitated in TALP medium and eggs collected 14–16 h after hCG injection. The results show that in these mice TALP will support sperm motility and *in vitro* capacitation, as in hamster spermatozoa. The hamster IVF procedure was established using a defined medium containing taurine–hipotaurine, identified in sperm extracts from various hamster tissues (Bavister *et al.*, 1978; Meizel *et al.*, 1980; Bavister, 1989). Mrsny *et al.* (1979) and Meizel *et al.* (1980) reported that taurine and hipotaurine might play a role in the maintenance and stimulation of sperm motility and in the stimulation of capacitation and the acrosome reaction *in vivo*. In *Calomys* spermatozoa, the type of medium was found to affect motility, which decreased with time from 30 to 120 min. The highest percentage of motility was observed when spermatozoa were incubated with TALP, irrespective of the sperm concentration. For both *Calomys* species, at the same sperm concentration, TALP supported motility better than

T6 medium for up to 120 min of incubation. In general, the time for capacitation in a culture medium varies among rodent species. For example, 1–2 h is required for mouse spermatozoa (Toyoda *et al.*, 1971), 2.5 h for hamster spermatozoa (Yanagimachi, 1970), 5–7 h for rat spermatozoa (Niwa and Chang, 1974) and 8–12 h for guinea-pig spermatozoa (Yanagimachi, 1972). In the present study, *C. musculus* spermatozoa were able to penetrate homologous eggs after 90 min and *C. laucha* spermatozoa did so after 60 min. *In vitro* capacitation of mouse spermatozoa under adequate conditions is known to bring about hyperactivation (Yanagimachi, 1970) and for *Calomys* hyperactivated spermatozoa of both species incubated in TALP medium were characterized by a distinct 'circular' movement first observed at 30 min. Non-hyperactivated spermatozoa showed planar motility, as observed during incubation in T6 medium. The 'figure-of-eight' pattern described for capacitated mouse spermatozoa (Yanagimachi, 1970; Suarez, 1988) was not seen in *Calomys* spermatozoa.

The spermatozoa of *Calomys*, like those of other mammals, undergo the acrosome reaction before penetrating the zona pellucida. In the present study, the acrosome reaction was visible under the light microscope in both the hooked heads of *C. musculus* spermatozoa and the hookless-heads of *C. laucha* spermatozoa. In both species, a maximum number has reacted at 90 min of incubation. However, for IVE, a 60 min period of sperm pre-incubation was chosen to avoid insemination of a population of > 50% acrosome-reacted spermatozoa.

The results presented here indicate that TALP is a suitable medium for *Calomys* spermatozoa in regard to capacitation, the acrosome reaction, and for the hamster test, which confirms the state of capacitated spermatozoa and their ability to fuse with, and be incorporated into, the oocyte. For both *Calomys* species, the maximum percentage of fertilized eggs was obtained after pre-incubation with 10^7 spermatozoa ml^{-1} and insemination *in vitro* with 10^6 spermatozoa ml^{-1} . These results confirm that TALP medium and a concentration of 10^7 spermatozoa ml^{-1} for pre-incubation are appropriate for capacitation leading to fertilization *in vitro*. Moreover, in both species, optimal percentages for hyperactivation were obtained for this medium at 60 min of incubation and 10^7 spermatozoa ml^{-1} . Furthermore, these conditions produced only 20–30% of spontaneously reacted spermatozoa at the time chosen for *in vitro* insemination; the percentage of acrosome-reacted spermatozoa subsequently increased up to 120 min of incubation.

In the last two decades the characterization of gametes in small rodents such as the mice, rats and hamsters has advanced considerably. At present, these species are commonly used as models for reproductive studies. However, mammalian fertilization is a complicated process, and understanding of the basis of species-specificity and the molecular mechanisms underlying the whole reproductive process will require evidence from a wider variety of species. It is expected that *C. musculus* and *C. laucha* will be useful laboratory animals from which to gain further understanding of mammalian reproduction.

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